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Award Number: DAMD17-00-1-0600

TITLE: A Novel Strategy to Inhibit Osteolytic Bone Metastases of Breast
Cancer

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REPORT DATE: May 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2002	Final (1 Oct 00 - 1 Apr 02))

4. TITLE AND SUBTITLE
A Novel Strategy to Inhibit Osteolytic Bone Metastases of Breast Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0600

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
More than 70% of women who die from breast cancer show osteolytic bone metastases which cause significant morbidity. Therefore, inhibition of osteolytic bone metastasis would improve the quality of life of patients with advanced breast cancer. Rational therapies to prevent osteolytic bone metastasis of breast cancer should be based on the unique characteristics of the bone microenvironment. The development and progression of osteolytic bone metastases are dependent on the resorption of bone by osteoclasts, multinucleated giant cells that originate from hematopoietic stem cells of the macrophage/monocyte lineage. Differentiation of osteoclasts is absolutely dependent on cell-to-cell contact between osteoblasts/stromal cells and hematopoietic cells. Of critical importance is a membrane-bound cytokine expressed in osteoblasts/stromal cells: RANKL (receptor activator of NF-κB ligand). RANKL binds to RANK, a cell-associated protein present on hematopoietic cells, thereby controlling the development and activation of osteoclasts. In this Concept Award, we proposed to downregulate the expression of RANKL by osteoblasts. Functional knockout of RANKL was accomplished by the intracellular expression of an anti-RANKL single-chain antibody. We hypothesize that this will inhibit the recruitment and activation of osteoclasts, thereby reducing osteoclastic bone resorption.

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)
Clinical and Experimental Therapeutics; Breast Cancer; Bone; Gene Therapy

15. NUMBER OF PAGES
10

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	**Unlimited**

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INTRODUCTION

More than 70% of women who die from breast cancer show osteolytic bone metastases that cause significant morbidity, including pain, pathological fractures, hypercalcemia and nerve compression. Therefore, inhibition of osteolytic bone metastasis would improve the quality of life of patients with advanced breast cancer. Rational therapies to prevent osteolytic bone metastasis of breast cancer should be based on the unique characteristics of the bone microenvironment. In this regard, the development and progression of osteolytic bone metastases are dependent on the resorption of bone by osteoclasts, multinucleated giant cells that originate from hematopoietic stem cells of the macrophage/monocyte lineage. Differentiation of osteoclasts is absolutely dependent on cell-to-cell contact between osteoblasts/stromal cells and hematopoietic cells. Of critical importance is a membrane-bound cytokine expressed in osteoblasts/stromal cells: RANKL (receptor activator of NF-kappaB ligand). RANKL binds to RANK, a cell-associated protein present on hematopoietic cells, thereby controlling the development and activation of osteoclasts. We hypothesize that downregulation of the expression of RANKL by osteoblasts will inhibit the recruitment and activation of osteoclasts, thereby reducing osteoclastic bone resorption. To this end, we chose to accomplish functional knockout of RANKL within osteoblasts by the intracellular expression of an anti-RANKL single-chain antibody.

BODY

We hypothesize that downregulation of the expression of receptor activator of NF-kappaB ligand (RANKL) by osteoblasts will inhibit the recruitment and activation of osteoclasts, thereby reducing osteoclastic bone resorption [1] and inhibiting osteolytic bone metastases of breast cancer. To this end, we chose to accomplish functional knockout of RANKL within osteoblasts by the intracellular expression of an anti-RANKL single-chain antibody. The variable region (Fv) portion of an antibody is comprised of the antibody heavy and light (V_H and V_L) domains and is the smallest antibody fragment containing a complete antigen-binding site. A recombinant Fv can be expressed in a single-chain construct (scFv) by connecting the V_H and V_L domains by a short peptide linker.

The first specific aim was to derive a single-chain antibody (scFv) against RANKL. A BALB/c mouse was immunized according to standard techniques with soluble human RANKL (Research Diagnostics, Inc.), a 20 kDa protein comprising the full-length of the extracellular domain of RANKL. The spleen was harvested, mRNA isolated and cDNA synthesized using commercial kits. The cDNA served as the template for the amplification of the immunoglobulin V_H and V_L chains by the polymerase chain reaction (PCR) using a set of primers that has been empirically tested and evolved over more than 7 years [2]. The PCR products were then cloned into the phage-display vector pSEX81 to produce an scFv library [3]. *E. coli* were transformed with the recombinant phagemid and infected with a helper M13 phage to yield recombinant phage that display scFvs fused to the minor coat protein, pIII. Phage-displayed scFvs that bind to RANKL were enriched from the library by successive rounds of panning against immobilized recombinant RANKL [4]. Nonspecific phages were removed during the washing step, after which the bound phages were eluted and used to reinfect *E. coli*. After five rounds of panning, the specificity of the enriched phage particles for RANKL was confirmed by screening 96 individual clones in an enzyme-linked immunosorbent assay (ELISA). Phage bound to immobilized RANKL were detected by a mouse anti-M13 monoclonal antibody (mAb) conjugated to horseradish peroxidase. ABTS was employed as the chromogenic substrate and absorbance was measured at 405 nm. As shown in Fig. 1, clone H10 showed a high affinity for RANKL.

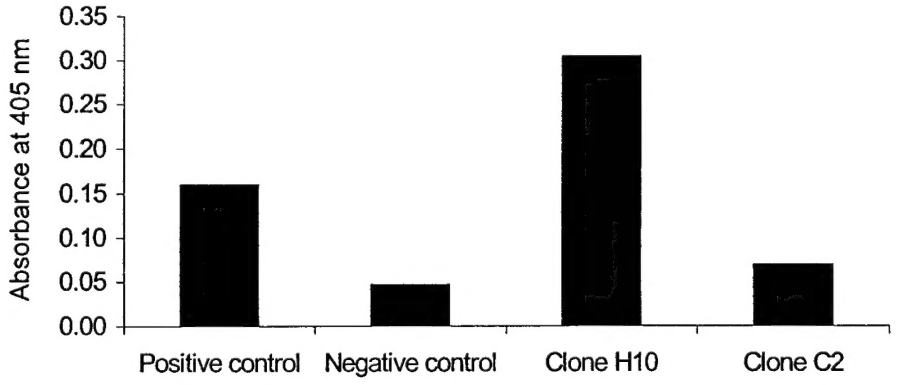

Fig. 1. ELISA to detect phage-displayed anti-RANKL scFv.

Having identified an anti-RANKL scFv, we subcloned the cDNA into a plasmid vector (pCMV/myc/ER; Invitrogen) designed to target proteins to the endoplasmic reticulum (ER), a subcellular compartment through which RANKL must traffic during the process of maturation. To this end, the signal peptide from a mouse V_H chain directs the scFv protein to the ER, and the SEKDEL sequence at the C-terminus leads to its retention in the ER. The expressed scFv was also tagged at the N-terminal with the c-myc epitope to facilitate detection of the recombinant protein. To confirm that the anti-RANKL scFvs can be expressed in mammalian cells, HeLa cells were transfected using the Superfect transfection reagent (Qiagen). Forty-eight hours post-transfection, the cells were lysed and immunoblot analysis performed using an anti-myc mAb (mAb 9E10; Invitrogen). Expression of the anti-RANKL scFv was indicated by the recognition of a 34 kDa protein by the anti-myc mAb.

We next sought to examine whether the anti-RANKL scFv could reduce the level of RANKL in osteoblasts. The human MG63 and Saos-2 osteoblast-derived osteosarcoma cell lines, which have previously been shown to express RANKL [5], were transfected with the plasmid expressing the ER-directed anti-RANKL scFv. The cells were maintained in the absence or presence of 10 nM parathyroid hormone 1-34 (Sigma) to stimulate the expression of RANKL. Forty-eight hours post-transfection, immunoblot analysis was performed to ascertain whether the ER-targeted scFv downregulates expression of RANKL in these cell lines. Mock-transfected cells and cells transfected with a plasmid encoding an irrelevant scFv served as controls. Although we employed anti-RANKL antibodies from three distinct commercial sources, we were unable to detect a specific band in the immunoblot corresponding to RANKL. Thus, we had to modify our approach.

We therefore constructed a plasmid encoding sRANKL with an N-terminal FLAG epitope tag to permit its detection. HeLa cells were cotransfected with the plasmid expressing FLAG-RANKL and the plasmid expressing the myc-tagged, ER-targeted anti-RANKL scFv. An irrelevant scFv was employed as a control. Forty-eight hours post-transfection, immunoblot analysis using an anti-FLAG mAb as primary antibody was performed to ascertain whether the scFv downregulates expression of FLAG-RANKL. Detection using an anti-myc mAb indicated that the equivalent amounts of the anti-RANKL scFv were employed in all cases. As shown in Fig. 2, expression of the ER-targeted anti-RANKL scFv led to a downregulation of the level of RANKL.

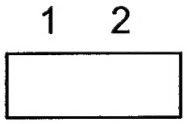

Fig. 2. The anti-RANKL scFv downregulates expression of FLAG-RANKL. HeLa cells were cotransfected with FLAG-RANKL and an irrelevant scFv (lane 1) or the anti-RANKL scFv (lane 2). Forty-eight hours post-transfection, the cells were lysed and equivalent amounts of cellular protein were subjected to immunoblot analysis. An anti-FLAG mAb was used as the primary antibody.

KEY RESEARCH ACCOMPLISHMENTS

- Generation of an intracellular single-chain antibody that selectively downregulates the expression of RANKL.

REPORTABLE OUTCOMES

The data generated in the work supported by this award will form the basis of an application for a DOD Breast Cancer Research Program Idea Award.

CONCLUSIONS

During this award period, we generated an intracellular single-chain antibody that selectively downregulates the expression of RANKL. We are now initiating experiments to determine whether the downregulation of RANKL in osteoblasts results in a reduction in osteoclast recruitment and activation in vitro. To this end, we will perform an osteoclast formation assay using a system that models the interaction of pre-osteoclastic macrophages with the osteoblast. MG-63 osteosarcoma cells or stro-1(+) cells will be transfected with vectors expressing either the anti-RANKL sFv or an irrelevant sFv. These transfected osteoblastic cells will be cocultured with osteoclast precursors from the Human Bone Cell Core Facility. After 3, 5, 7, 10 and 14 days, we will assay two osteoclast-specific markers, tartrate-resistant acid phosphatase (TRAP) and cathepsin K. It is hypothesized that the expression of the anti-RANKL sFv will downregulate RANKL on the osteoblast cell membrane, preventing its interaction with RANK on the surface of osteoclasts and thereby inhibiting osteoclastogenesis. This would result in a decrease in the number of TRAP- and cathepsin K-positive cells, compared to the controls. We will then investigate the ability of the anti-RANKL sFv to inhibit the resorption of bone by osteoclasts. This will employ pit assays, measuring the formation of resorption pits on slices of whale dentin, or degradation of radiolabeled bone fragments.

The successful inhibition of osteoclast recruitment and activation in vitro would warrant future studies to evaluate the efficacy of this novel strategy in the treatment of osteolytic breast cancer metastases. To this end, it would be necessary to generate a gene delivery vector capable of directing the efficient expression of the anti-RANKL sFv specifically to osteoblasts. In vivo studies would be performed using a murine model in which the intracardiac injection of a human breast cancer cell line, MDA-MB-231, leads to osteolytic bone metastases.

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